Linkage of Subunit Interactions, Structural Changes, and Energetics of Coenzyme Binding in Tryptophan Synthase†

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ABSTRACT: The energetics of binding of the coenzyme pyridoxal 5′-phosphate (PLP) to both the apo β_2 subunit and the apo $\alpha_2\beta_2$ complex of tryptophan synthase from *Escherichia coli* has been investigated as a function of pH and temperature by direct microcalorimetric methods. At 25 °C, pH 7.5, the binding process proceeds in the time range of minutes and shows a biphasic heat output which permits resolution of the overall reaction into different reaction steps. Binding studies on the coenzyme analogues pyridoxal (PAL), pyridoxine 5′-phosphate (PNP), and pyridoxine (POL) to the protein as well as a comparison of these results with data from studies on PLP binding to ϵ-aminocaproic acid have led to a deconvolution of the complex heat vs. time curves into fast endothermic contributions from electrostatic interaction and Schiff base formation and slow exothermic contributions from the interactions between PLP and the binding domain. The pH-independent, large negative change in heat capacity of about −9.1 kJ/(mol of β_2·K) when binding PLP to β_2 is indicative of major structural changes resulting from complex formation. The much smaller value of ΔC_p = −1.7 kJ/(mol of β_2·K) for binding of PLP to $\alpha_2\beta_2$ clearly demonstrates the energetic linkage of protein–protein and protein–ligand interactions. Calorimetric titrations of the apo β_2 subunit with PLP at 35 °C have shown that also at this temperature positive cooperativity between the two binding sites occurs. On the basis of these measurements a complete set of site-specific thermodynamic parameters has been established. Due to the slow calorimetrically observable rate of the overall binding reaction and the occurrence of enthalpic processes separated on the time scale, it has been possible to infer a binding mechanism. According to this analysis the reaction consists of a minimum of three identifiable steps. This conclusion is in excellent agreement with results of a kinetic study based on UV and fluorescence quenching experiments [Bartholmes, P., Balk, H., & Kirschner, K. (1980) *Biochemistry 19*, 4527–4533].

The $\alpha_2\beta_2$ bienzyme complex of tryptophan synthase from *Escherichia coli* is one of the few examples where linkage between subunit interaction and ligand binding has been studied [for a recent review, see Miles (1979)]. However, until recently the only quantitative thermodynamic data available for this enzyme were Gibbs energies, $\Delta G°$. Since $\Delta G°$ values are particularly difficult to interpret in molecular terms (Hinz, 1983), calorimetric binding studies on the association of α and β_2 subunits of the enzyme have been performed (Wiesinger et al., 1979). One of the results of these investigations has been the finding that the presence of the coenzyme pyridoxal 5′-phosphate (PLP) on the β_2 subunit drastically changes both the enthalpy of subunit interaction and its temperature dependence. An alternative, but equivalent, effect of ligand binding on these interactions can be obtained by comparing the energetics of binding of PLP to the native tetramer and to the isolated β_2 subunit. It has been shown by equilibrium studies that both the isolated β_2 subunit and the $\alpha_2\beta_2$ complex bind two molecules of PLP. The reaction involves the covalent aldimine linkage to the ϵ-amino group of lysine-86 in the active center of the β chain (Crawford et al., 1980) as well as noncovalent interactions between the various groups of the coenzyme and the binding domain of the protein. Binding of PLP to the isolated β_2 subunit is cooperative with dissociation constants of 8.7×10^{-6} M and 2.3×10^{-7} M at 20 °C, pH 7.5, in 0.1 M sodium pyrophosphate buffer for the first and second site, respectively, while binding to the sites of the $\alpha_2\beta_2$ complex is noncooperative and characterized by a value of the dissociation constant of 1.0×10^{-6} M (Bartholmes et al., 1976). A sequence of three steps of decreasing rate has been inferred from kinetic studies for the association between PLP and β_2: (a) formation of a noncovalent complex between coenzyme and enzyme; (b) isomerization to an enzymatically inactive internal aldimine; (c) formation of the active holoenzyme (Bartholmes et al., 1980). Preliminary calorimetric studies have shown that complex formation between PLP and the β_2 subunit is characterized by a slow, strongly pH-dependent biphasic heat output which originates from at least two different molecular events, which have reaction enthalpies of opposite sign (Wiesinger & Hinz, 1980). The present studies have been undertaken to deconvolute the complex overall reaction enthalpy into ΔH values, which can be attributed to well-defined molecular contributions. They involve measurements of the association of PLP and the PLP analogues pyridoxal (PAL), pyridoxine 5′-phosphate (PNP), and pyridoxine (POL) with the isolated β_2 subunit and the $\alpha_2\beta_2$ complex as a function of both temperature and pH.

Materials and Methods

The α and β_2 subunits of tryptophan synthase were purified and stored as described previously (Kirschner et al., 1975; Bartholmes et al., 1976). An extinction coefficient of E (0.1%, 278 nm) = 0.46 and 0.58 (cm^2 mg^{-1}) was used for the α and apo β_2 subunit, respectively. The concentration of PLP was determined by using an extinction coefficient $E = 6.6 \times 10^3$ M^{-1} cm^{-1} at 388 nm. Apo β_2 protein was prepared by treating the holoenzyme with 0.1 M hydroxylamine on a Sephadex G-25 column; the tetrameric complex was reconstituted by adding an at least threefold excess of α subunits to a solution of the β_2 dimer (Wiesinger et al., 1979). All experiments were performed in 0.1 M sodium pyrophosphate buffer adjusted with HCl to the desired pH.

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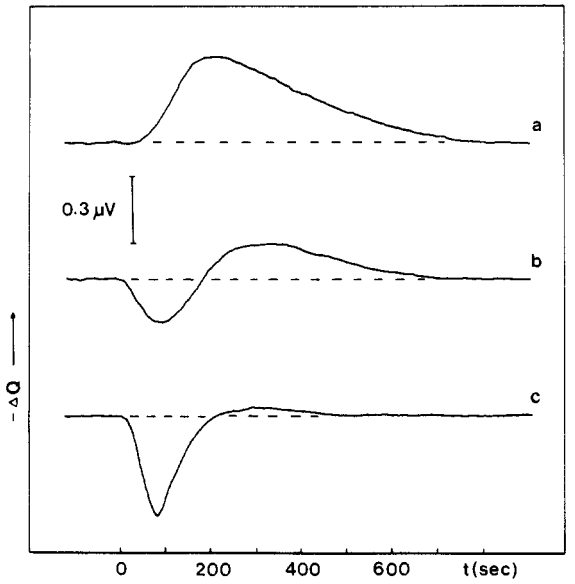

FIGURE 1: Calorimetric records of the heat effect ΔQ as a function of time for the reaction between the apo β_2 subunit and PLP at 25 °C at pH 8.0 (a), pH 7.5 (b), and pH 7.0 (c). Pulse duration: 50 s (0.37 mL). $[\beta_2] = 5.8 \times 10^{-5}$ M; $[PLP] = 7.9 \times 10^{-5}$ M after mixing the solutions 1:1 in the calorimeter.

PLP was purchased from Serva, Heidelberg. All other chemicals were obtained from Merck, Darmstadt, and were of reagent grade quality. PNP was prepared by treating PLP with sodium borohydride (Peterson & Sober, 1954) and was a gift of Dr. Hubert Balk, Regensburg. Absorption measurements were performed in a Varian Cary 118 spectrophotometer equipped with a thermostated cuvette holder; pH values were adjusted at the temperature of the experiment by using a digital pH meter (WTW, Weilheim). The microcalorimeter (Weber & Hinz, 1976) was operated in the pulsed flow mode with pulse duration between 20 and 50 s, corresponding to 0.15 and 0.37 mL of solution, respectively. All molar enthalpies have been referred to a molecular weight of 89 000 for the β_2 subunit and have been corrected for dilution effects of the components. The calorimeter was calibrated by using the well-known heats of neutralization of water and dilution of sucrose, respectively (Grenthe et al., 1970; Gucker et al., 1939). The error within a single determination of a ΔH value consisting of four to seven measurements is about 10% and is not listed in the tables.

Results

Binding of the Coenzyme to the Isolated β_2 Subunit as a Function of pH. The time-resolved heat output observed on binding of PLP to the apo β_2 subunit at 25 °C is shown for different pH values in Figure 1. The overall reconstitution process as seen in the calorimeter is slow and occurs in the time range of several minutes. The reaction enthalpy changes its sign as a function of pH; the process is apparently composed of at least two reactions with heat effects of opposite sign. The overall apparent ΔH values of the reaction which are calculated from the total area between the reaction curve and the base line, taking into account the proper signs, are given in Table I. Partition of the overall enthalpy into contributions from the different binding sites is not possible due to the high association constants. All calorimetrically feasible measurements require PLP concentrations well above the saturation level of both sites as can be calculated from the association constants. These constants were estimated by using the experimental values at 20 °C of Bartholmes et al. (1976) and applying the van't Hoff equation. The pH dependence of the overall apparent reaction enthalpies is shown in Figure 2.

Table I: Overall Apparent ΔH Values for the Reaction of PLP with the Apo β_2 Subunit at 25 °C in 0.1 M Sodium Pyrophosphate Buffer

pH	ΔH (kJ/mol of β_2)	pH	ΔH (kJ/mol of β_2)
6.8	35.8 ± 3.3	7.8	−22.2 ± 0.6
7.0	16.5 ± 1.4	8.0	−31.7 ± 4.2
7.3	−1.9 ± 2.6	8.2	−48.7 ± 11.0
7.5	−15.3 ± 0.8		

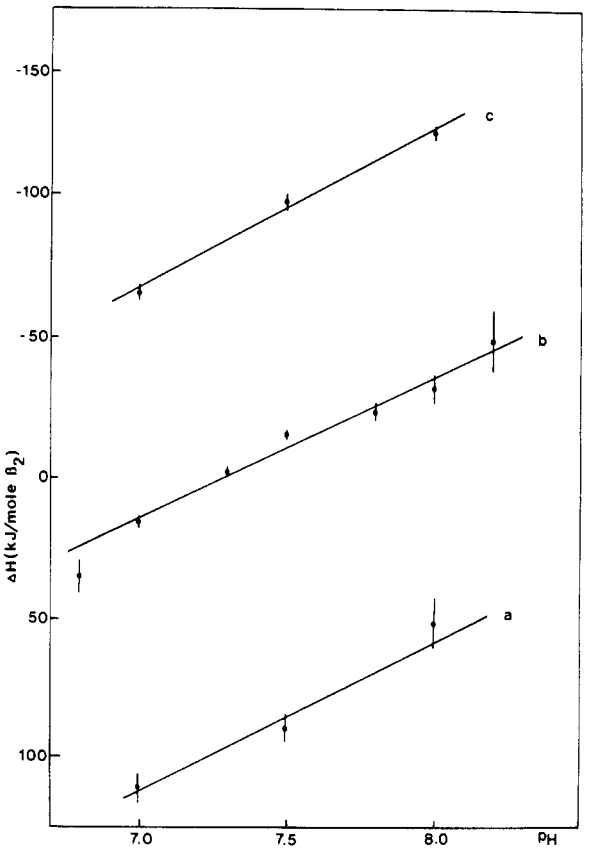

FIGURE 2: pH dependence of the overall apparent binding enthalpies of PLP to the β_2 subunit at 15 (a), 25 (b), and 35 °C (c). Error limits are expressed as standard deviation.

Studies on the PLP Analogues. In order to sort out the contributions of the various groups of the coenzyme to the overall binding enthalpy, microcalorimetric studies were carried out with the three PLP analogues PAL, PNP, and POL. PAL lacks the 5′-phosphate group responsible for noncovalent attachment, PNP is reduced in the 4-position and cannot form the covalent aldimine linkage, and POL has neither the carbonyl function nor the phosphate moiety. Representative calorimetric experiments are shown in Figure 3. Binding of PNP to the isolated apo β_2 subunit showed only one fast endothermic process. At high PNP concentrations (3×10^{-3} M) and pH 7.5 a saturation enthalpy of 14.5 kJ/mol of β_2 could be determined for binding of two PNP molecules; variation of the PNP concentration did not result in changes of the reaction enthalpy. This finding is consistent with the result of calculations which showed that, by use of the association constants reported by Tschopp & Kirschner (1980), saturation of more than 90% is achieved at 3 mM PNP.

In contrast to the fast endothermic association of the apo β_2 protein and PNP, the reaction of PAL with the enzyme is slow and endothermic. Only after a time lag of about 50 s can a heat effect be detected in the calorimeter (Figure 3). No equilibrium constants are known for this reaction, and titration experiments in the calorimeter did not yield saturation

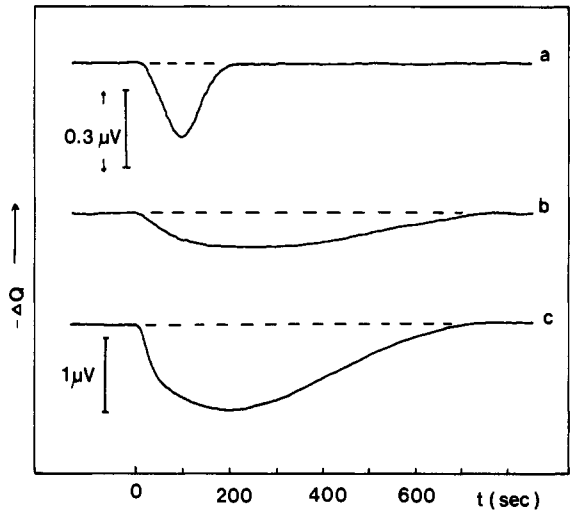

FIGURE 3: Calorimetric records of the heat effect ΔQ as a function of time at 25 °C, pH 7.5, for the reactions (a) apo β_2-PNP: $[\beta_2] = 4.2 \times 10^{-5}$ M; [PNP] = 7.6×10^{-4} M. (b) apo β_2-PAL: $[\beta_2] = 4.9 \times 10^{-5}$ M; [PAL] = 9.2×10^{-3} M. (c) AC–PAL: [AC] = 3.6×10^{-2} M; [PAL] = 2.9×10^{-2} M. Pulse duration: 50 s (0.37mL). AC = ϵ-aminocaproic acid. Values refer to concentrations after mixing.

enthalpy values even at such high ligand concentration as 10 mM. However, the enthalpies obtained at different PAL concentrations can be used to estimate an equilibrium constant. The approximate dissociation constant resulting from this treatment is 0.1 M. This value is comparable with the dissociation constant of 0.3 M obtained for the interaction between PAL and ϵ-aminocaproic acid at 25 °C, pH 7.5, in the same buffer (H. Wiesinger and H.-J. Hinz, unpublished results). When the value 0.1 M is used for the dissociation constant, a saturation enthalpy ΔH = 105 kJ/mol of β_2 can be estimated for complex formation between β_2 and PAL. Qualitatively, the reactions of PAL and PNP with the enzyme differ by their dependence of ΔH on pH. The heat uptake observed when β_2 is mixed with PAL by using identical nonsaturating concentrations at pH 7.0, 7.5, and 8.0 increases with increasing pH while the ΔH values obtained for complex formation between β_2 and PNP decrease with increasing pH. To evaluate the contributions of groups other than the phosphate moeity and the carbonyl group of PLP, experiments have been performed employing POL. However, even at 10^{-2} M POL no heat effect could be observed at pH 7.0 and 7.5 when the compound was mixed with the apo β_2 subunit. Although in principle this could mean that the reaction is athermic, the more likely interpretation of the result is that no complex formation takes place even at high ligand concentrations.

Variation with Temperature of the Binding Enthalpy of PLP to the Isolated β_2 Subunit. An important parameter for characterization of structural changes associated with binding of ligands to biological macromolecules is the variation with temperature of the reaction enthalpies (Sturtevant, 1977; Hinz, 1983). Therefore, ΔH values for complex formation between PLP and the apo β_2 subunit were also determined at 15 and 35 °C. At 15 °C β_2 was saturated with PLP at all concentrations employed, as was shown by the independence of the ΔH value of ligand concentration. In accordance with van't Hoff calculations based on the calorimetrically obtained enthalpies a much weaker interaction between β_2 and PLP was observed at 35 °C. Saturation at this temperature is only attainable at 2 mM PLP.

The results of the measurements at 15 and 35 °C are summarized in Table II and Figure 4 where saturation enthalpies at pH 7.0, 7.5, and 8.0 are plotted as a function of temperature.

Table II: Temperature Dependence of the Apparent Reaction Enthalpies of PLP with the Apo β_2 Subunit in 0.1 M Sodium Pyrophosphate Buffer

T (°C)	pH	ΔH (kJ/mol of β_2)
15	7.0	118.8 ± 7.5
	7.5	90.9 ± 4.2
	8.0	50.7 ± 12.1
35	7.0	−66.2 ± 2.5
	7.5	−98.4 ± 4.2
	8.0	−122.7 ± 2.9

Table III: Thermodynamic Parameters of PLP Binding to the Apo β_2 Subunit at 35 °C in 0.1 M Sodium Pyrophosphate Buffer[a]

	at pH		
	7.0	7.5	8.0
$K_{D,1}$ (M) × 10^5	3.1	4.0	7.9
$K_{D,2}$ (M) × 10^5	1.2	2.0	6.0
$\Delta G_{D,1}$ (kJ/mol)	26.9	25.9	24.4
$\Delta G_{D,2}$ (kJ/mol)	29.4	27.6	25
$\Delta H_{D,1}$ (kJ/mol)	34.9	56.4	68
$\Delta H_{D,2}$ (kJ/mol)	31.5	42	55
$\Delta S_{D,1}$ (J/mol·K)	25.2	96.6	142.8
$\Delta S_{D,2}$ (J/mol·K)	8.4	46.2	96.6

[a] The ranges within which the parameters can be varied without a significant aberration of the calculated curve from the experimental values are the following: $K_D = \pm 0.5 \times 10^{-5}$; $\Delta H_D = \pm 3.9$ kJ/mol. The parameters refer to the dissociation reaction and to 1 mol of binding site.

Table IV: Apparent Reaction Enthalpies and Their Temperature Dependence for the Reconstitution of Apo $\alpha_2\beta_2$ with PLP in 0.1 M Sodium Pyrophosphate Buffer

T (°C)	pH	ΔH (kJ/mol of β_2)
15	7.5	−0.8 ± 2.9
25	7.0	3.3 ± 0.8
	7.5	−16.5 ± 2.5
	8.0	−25.1 ± 0.4
35	7.5	−33.9 ± 0.8

At all three pH values binding enthalpies become more negative with increasing temperature. Linear regression analysis results in the following equations, where t refers to the temperature in degrees Celsius.

$$\text{pH 7.0} \quad \Delta H = -8.9t + 243 \text{ kJ/mol of } \beta_2 \quad (1)$$

$$\text{pH 7.5} \quad \Delta H = -9.4t + 229 \text{ kJ/mol of } \beta_2 \quad (2)$$

$$\text{pH 8.0} \quad \Delta H = -8.9t + 182 \text{ kJ/mol of } \beta_2 \quad (3)$$

Within experimental uncertainty the heat capacity change can be considered identical for the three pH values studied. The average value is $\Delta C_p = -9.1 \pm 0.6$ kJ/(mol of β_2·K).

Thermal Titrations of β_2 with PLP at 35 °C at Various pH Values. Due to the larger dissociation constant, calorimetric binding studies could be performed to characterize the binding equilibrium between PLP and β_2 at 35 °C. Figure 5 shows three curves at 35 °C and pH 7.0, 7.5, and 8.0, respectively. Each point constitutes the average ΔH values of three to four measurements. The curves have been calculated on the basis of the dissociation constants, and the enthalpies for each binding site given in Table III. These parameters have been obtained from iterative least-squares procedures using the experimentally determined overall saturation enthalpies listed in Table II and treating the K_D values and site-specific enthalpies as adjustable parameters in a sequential binding model of the Adair type (Wiesinger et al., 1979).

PLP Binding to the Apo $\alpha_2\beta_2$ Complex. Mixing of PLP with $\alpha_2\beta_2$ at 25 °C results in a pronounced biphasic calorimetric trace analogous to that of the reaction of PLP with β_2

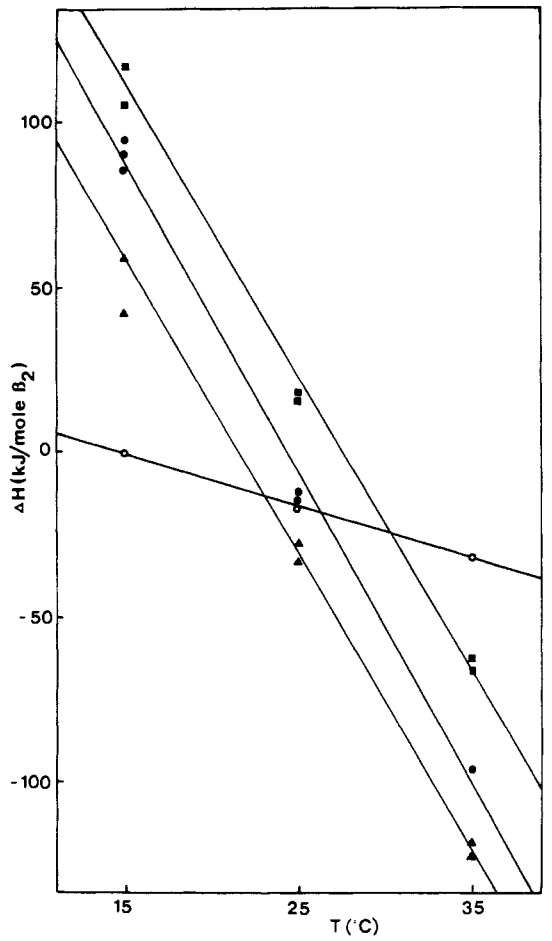

FIGURE 4: Temperature dependence of the binding enthalpy of PLP to the apo $\alpha_2\beta_2$ complex at pH 7.5 (○) and to the apo β_2 subunit of tryptophan synthase at pH 7.0 (■), pH 7.5 (●), and pH 8.0 (▲). The curves were drawn according to the linear regression analysis described in the text.

(Figure 1). The ΔH values obtained from calorimetric measurements at different temperatures are compiled in Table IV. Linear regression analysis yields the following equation for ΔH as a function of temperature:

$$\text{pH } 7.5 \qquad \Delta H = -1.7t + 24.7 \text{ kJ/mol of } \beta_2 \qquad (4)$$

ΔH is the association enthalpy, and t refers to the temperature in degree Celsius. The temperature coefficient $\Delta C_p = -1.7 \pm 0.2$ kJ/(mol of β_2·K) is considerably smaller than the value found for the binding of PLP to the isolated β_2 subunit (Figure 4). As a result of the changes in heat capacity that characterize binding of the coenzyme to both the β_2 subunit and $\alpha_2\beta_2$ complex, the biphasic shape of the calorimetric traces for a given pH value is less prominent at 15 and 35 °C than at 25 °C. At low temperature the overall enthalpy is controlled by endothermic contributions while at high temperature exothermic contributions are dominant.

Discussion

Complex Formation between PLP and β_2 at 25 °C: Decomposition of the Overall Enthalpy Change into Group Contributions. Usually the most accurate method to determine energetic interaction parameters in biochemical systems is direct calorimetric measurement of the heat effects involved in the reaction, since no mechanistic model is needed for the evaluation of the enthalpy values. Interpretation of the ΔH values in terms of molecular models or separation of the overall ΔH into contributions from different groups of the ligand is, however, generally very difficult, particularly for complex

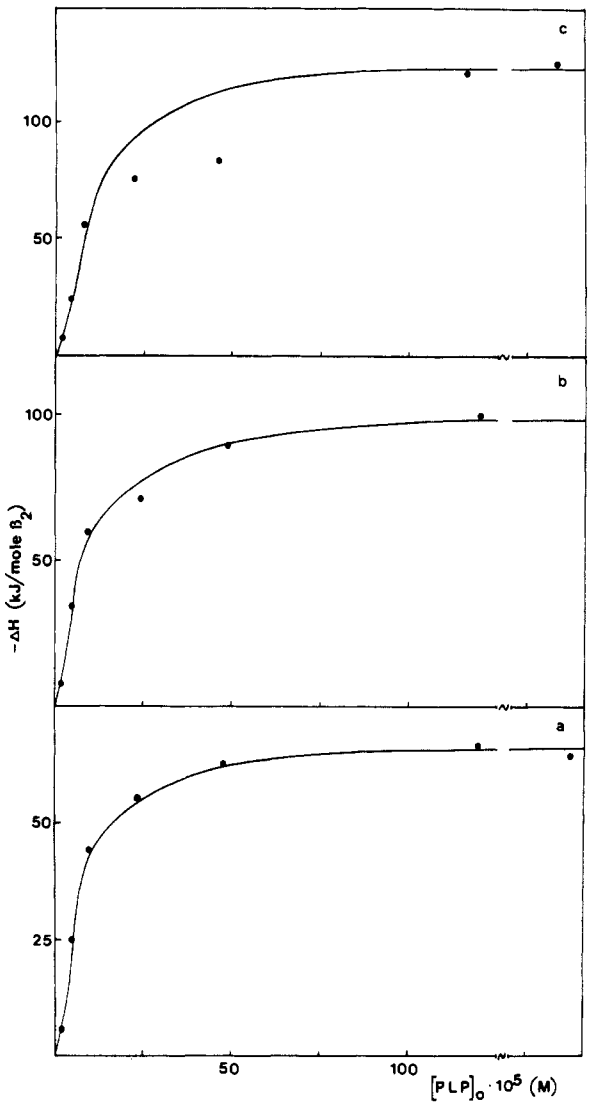

FIGURE 5: Enthalpimetric titration of the apo β_2 subunit of tryptophan synthase with PLP at 35 °C. (a) pH 7.0; $[\beta_2] = 4.2 \times 10^{-5}$ M. (b) pH 7.5; $[\beta_2] = 4.4 \times 10^{-5}$ M. (c) pH 8.0; $[\beta_2] = 5.9 \times 10^{-5}$ M. The curves have been calculated on the basis of the dissociation constants and enthalpy values given in Table III; values refer to concentrations after mixing.

reaction mechanisms (Niekamp et al., 1980). One promising way to partition an overall reaction enthalpy into group contributions appears to rely on measurements involving chemically modified ligand analogues. This approach has been followed in the present study. PLP analogues with systematically varied side groups have been employed. The heat vs. time curve monitored calorimetrically for binding of PLP to the apo β_2 subunit (Figure 1) exhibits a complex pattern. At least two processes can be resolved in time, a fast endothermic reaction and a much slower exothermic reaction lasting up to 8 min. In order to identify contributions resulting from covalent Schiff base formation and from noncovalent interactions, association of the enzyme with PNP, PAL, and POL has been investigated. The fast endothermic part visible in the reaction pattern of PLP with β_2 can be attributed to the interaction of the phosphate group with basic residues in the protein, probably Lys-75 or Arg-76, as suggested by Higgins et al. (1980). This assignment is supported by the completely analogous heat vs. time curve observed for binding of PNP, which, in comparison to PLP, lacks only the reactive carbonyl group to form the Schiff base. A strong electrostatic interaction between PLP in its dianionic form via a rigid salt bridge

to positively charged residues in the active center of the β protein was also postulated by Schnackerz & Bartholmes (1983) from the results of phosphorus-31 nuclear magnetic studies. The enthalpy contribution resulting from interaction of the phosphate group with the β_2 subunit as estimated from the PNP binding studies is +14.5 kJ/mol of β_2 or +7.3 kJ/mol of binding site. Values of similar magnitude have been observed for electrostatic interaction between small ions (Edsall & Wyman, 1958). The positive sign of the ΔH value is also consistent with the idea that electrostatic interactions in aqueous solution are often entropy driven. The lack of binding of POL even at 10^{-2} M concentration, which is indicated by the absence of a reaction enthalpy in our calorimetric studies as well as by other techniques (Balk, 1981), is unfortunately no sound basis for separating the ΔH of PNP association, which represents the sum of the enthalpic contributions of the ring and the phosphate moiety, into two individual ΔH values. Although we favor the interpretation that there is zero contribution to ΔH from the ring system, we recognize that the mere absence of complex formation between POL and the enzyme is only suggestive evidence.

It is difficult to judge whether PNP binding to enzymes involves in general only electrostatic interaction via the phosphate group. The type of interaction probably depends on the enzyme systems. For example, binding of PNP to tyrosine aminotransferase is characterized by an enthalpy change of 57 kJ/mol as calculated by a van't Hoff analysis (Borri-Voltattorni et al., 1975). While this value has the same sign as the ΔH determined by us calorimetrically for PNP interaction with the β_2 subunit of tryptophan synthase, its magnitude appears to be too large as to be accounted for by electrostatic interaction alone.

The second significant contribution to binding between PLP and the β_2 subunit stems from covalent Schiff base formation between the carbonyl group of the coenzyme and Lys-86 of the enzyme. To obtain energy parameters for this interaction, calorimetric studies have been performed on binding of PAL to the β_2 subunit and to ϵ-aminocaproic acid. The latter compound was chosen to mimic Schiff base formation with a lysine group of the enzyme. A comparison between the calorimetric curves of both reactions (Figure 3) shows that either reaction is slow and is associated with a positive enthalpy change. From these results it can be concluded that formation of an imine linkage in model compounds and in the enzyme exhibits the same energetic pattern. However, the magnitude of the interaction enthalpies is different for the two processes. Schiff base formation between PAL and ϵ-aminocaproic acid absorbs 22 kJ/mol, while the ΔH values for the same reaction with β_2 subunit is 105 kJ/mol of β_2 or approximately 53 kJ/mol of β. The difference between these enthalpies can easily be rationalized by the following considerations. While PLP is specifically attached to the active center of β_2 via the phosphate group, PAL can actually react with any accessible lysine group of the subunit. It is known that there are 18 lysine residues per β chain. From our experiments we cannot distinguish whether PAL has reacted with the lysine in the active center or with two accessible lysine residues on the surface of the β chain. However, it is probable that the intrinsic ΔH value for any covalent imine formation is the same. Therefore, it is rather likely that the larger enthalpy value observed for Schiff base formation between PAL and β_2 does not reflect an intrinsically higher interaction enthalpy but rather the fact that approximately two imine linkages are formed per β chain.

At 25 °C, pH 7.5, the two positive contributions to the overall ΔH of PLP binding, which result from electrostatic interaction of the phosphate group and from covalent Schiff base formation, are contained in the fast endothermic part of the calorimetric heat vs. time curve. This assignment is supported by spectrophotometric studies of the kinetics of Schiff base formation between ϵ-aminocaproic acid and PLP or PAL (H. Wiesinger and H.-J. Hinz, unpublished results). It could be shown that, even with ϵ-aminocaproic acid, PLP reacts faster than PAL. The difference in the activation parameters is due to blocking of the carbonyl function in PAL by formation of an internal cyclic hemiacetal (Ahrens et al., 1976). In the presence of the enzyme the rate of Schiff base formation of PLP relative to that of PAL is further increased by proper fixation of PLP via the phosphate group, which decreases the activation entropy of imine formation. Remembering that no heat effect was found when β_2 was mixed with high concentrations of POL, we can thus far conclude that only the ionic interaction between the phosphate moiety and cationic groups in the protein and the covalent Schiff base formation contribute significantly to the overall binding enthalpy of apo β_2 and PLP. Contributions from conformational changes in the enzyme will be discussed below.

Complex Formation between PLP and β_2 at 35 °C: Analysis of Thermal Titrations. It is not possible to split the overall enthalpies measured at 25 °C, pH 7.5, for binding of PLP to β_2 into contributions of the individual binding sites due to the high cooperativity of the reaction and the magnitude of the binding constants, which do not permit calorimetric studies under nonsaturating ligand concentrations. However, at 35 °C the affinity of the enzyme is lower, which renders it possible to perform thermal titrations with PLP. Curve fitting allows to extract site-specific enthalpies and Gibbs energies from the thermal titration curves.

The resulting sets of thermodynamic parameters for these pH values have been summarized in Table III. The two equilibrium constants and one ΔH value have been employed as adjustable parameters. The second ΔH value is given by the constraint that the sum of the two site-specific ΔH values is identical with the experimental overall enthalpy. Within the given error limits the sets of the thermodynamic parameters are unique. Some general features of the parameters are worth mentioning. The difference in affinity between the two binding sites for PLP obviously decreases with increasing temperature. At 20 °C, pH 7.5, the Gibbs energy difference between the first and the second binding site is 8.8 kJ/mol (Bartholmes et al., 1976) while at 35 °C a value of only 1.7 kJ/mol is observed. At all pH values the higher dissociation constant is associated with the larger enthalpy change, a result which was also found for the reaction between α subunits of tryptophan synthase and the apo β_2 subunit (Wiesinger et al., 1979). Independent of pH the Gibbs energy of binding is dominated by a strongly negative enthalpy. A slight decrease in affinity with increasing pH is due to the entropy contribution.

Dependence on pH of the Complex Formation between PLP and the β_2 Subunit. Association of PLP at 35 °C is accompanied by the uptake of approximately 0.6 proton per β chain from the solution, as can be calculated from the pH dependence of the $\Delta G°$ values (Alberty, 1969). The overall association enthalpy becomes more negative with increasing pH at all three temperatures as shown in Figure 2. Qualitatively, this variation of ΔH with pH can be rationalized by the assumption that neutralization of charged groups by protons occurs concomitantly with coenzyme binding. The proton uptake is consistent with such an interpretation of the data. It is, however, rather difficult to provide a more specific ex-

Table V: Thermodynamic Parameters of PLP Binding to the Apo $\alpha_2\beta_2$ Complex in 0.1 M Sodium Pyrophosphate Buffer, pH 7.5

T (°C)	$-\Delta G°_B$ (kJ/mol of bs)[a]	$-\Delta H°_B$ (kJ/mol of bs)[a]	$+\Delta S°_B$ [J/(mol of bs·K)][a]
15	33.6[b]	0.4	115.5
20	33.8	4.2[b]	100.8
25	34.2[b]	8.4	88.2
35	34.9[b]	16.8	58.8

[a] bs = binding site. [b] Values calculated from integrated van't Hoff equation (eq 5).

planation of the variation with pH of the enthalpies. Interaction of PLP with the β_2 subunit is a very complex phenomenon. Several ionic equilibria are perturbed by Schiff base formation, as can be demonstrated already for model compounds (H. Wiesinger and H.-J. Hinz, unpublished results). Shifts of pK values of the various ionizable groups on the PLP molecule occur as well as pK shifts affecting the imino proton of the Schiff base itself (Ahrens et al., 1970, Giartosio et al., 1982). Particularly enthalpic effects stemming from ionization of the phenolic oxygen and the ring nitrogen of PLP have to be considered. A dianion–monoanion transition of the phosphate moiety of the cofactor would, however, not contribute significantly to the overall enthalpy due to the low enthalpy of ionization. Such a process is also not very likely to occur in the pH range studied, since in the free coenzyme this ionization is associated with a pK of 6.2 and an enthalpy of 2 kJ·mol^{-1} (Giartosio et al., 1982). When bound to the enzyme, the pK is shifted to even lower values, as can be concluded from the phosphorus-31 nuclear magnetic resonance studies by Schnackerz & Bartholmes (1983), which showed that the ^{31}P chemical shift of PLP is pH independent between pH 6.7 and pH 8.5. Furthermore, besides the ionization processes, hydration of the carbonyl function of the coenzyme has been shown to be pH dependent (Johnson & Metzler, 1970). Therefore, at present it appears to be impossible to analyze the pH dependence of PLP binding to the β_2 subunit of tryptophan synthase in more detail.

Binding of PLP to the $\alpha_2\beta_2$ Apoenzyme. We have assumed that the noncooperative binding mechanism observed for association of two PLP molecules with the apo $\alpha_2\beta_2$ complex at 20 °C (Bartholmes et al., 1976) is also valid at 15 and 35 °C. Therefore, the experimental overall binding enthalpies have been equally partitioned among the two binding sites. By application of the integrated van't Hoff equation

$$\ln \frac{K_T}{K_{293}} = \frac{1}{R}\left(\frac{\Delta H_T}{T} - \frac{\Delta H_{293}}{293}\right) - \left(\frac{\Delta C_p}{R} \ln \frac{T}{293}\right) \quad (5)$$

(K_T, K_{293}, ΔH_T, and ΔH_{293} are the dissociation constants and enthalpies at T and 293 K, respectively; R is the gas constant, T is the absolute temperature in kelvin, and ΔC_p is the observed heat capacity change; ΔH_{293} can be calculated from eq 4 employing t = 20 °C). The standard Gibbs energies, $\Delta G°$, have been calculated for 15 and 35 °C from the equilibrium constant at 20 °C and the calorimetrically determined enthalpy values. The corresponding entropy changes have been estimated by using the Gibbs–Helmoholtz relationship. These thermodynamic parameters have been summarized in Table V. Inspection of the data shows that the stability of the holo $\alpha_2\beta_2$ complex does not vary significantly within the temperature range of the experiments. At low temperature the negative Gibbs energy results exclusively from the positive entropy change associated with binding of PLP; with increasing temperature the association enthalpy becomes more negative and contributes about 50% of the Gibbs energy at 35 °C. It is not possible to draw conclusions concerning the reaction mechanism on the basis of these variations in the thermodynamic parameters, since formally they can be explained as reflecting the change in heat capacity associated with PLP binding.

Comparison with Literature Data. ΔH values for coenzyme binding have been reported for two other PLP-dependent enzymes. In both cases the enthalpies have been derived from a van't Hoff analysis of binding studies. Complex formation of tyrosine decarboxylase with PLP is associated with an enthalpy change of −31 kJ/mol of binding site (Orlacchio et al., 1980), while the interaction of PLP and tyrosine aminotransferase involves a positive ΔH of 48 kJ/mol of binding site (Borri-Voltattorni et al., 1975). There is no explanation for the different sign of the ΔH in these two apparently similar reactions. However, it is worth noting that for both enzymes the binding enthalpies of PLP analogues have the same sign as the ΔH values for PLP. This result is in contrast to our findings for tryptophan synthase, where the ΔH for binding of PLP is different in sign from that for binding of PNP or PAL.

Recently calorimetric and potentiometric studies on binding of PLP to aspartate apoaminotransferase have been reported (Giartosio et al., 1982). Investigations at 19 and 25 °C in a pH range of 6.2–8.8 revealed a complex sigmoidal variation with pH of the binding enthalpy. This finding led to the assumption of the existence of two states of the enzyme, one at low and one at high pH, each of which is capable of binding PLP. Complex formation of PLP with the high pH state is characterized by an enthalpy which is more negative by approximately 25.1 kJ/mol than the corresponding ΔH for association of PLP with the low pH state. Analogous dramatic changes of the reaction enthalpy within one pH unit have been observed with tryptophan synthase (Figure 2).

Temperature Dependence of the Binding Enthalpy of PLP to Apo β_2 and $\alpha_2\beta_2$. A useful thermodynamic parameter to characterize structural changes of proteins associated with binding of ligands is the heat capacity change, ΔC_p (Hinz et al., 1971; Sturtevant, 1977; Hinz, 1983). It is most accurately determined from direct microcalorimetric binding studies at different temperatures. In comparison to Gibbs energies or enthalpies, heat capacity changes can be considered a more direct measure of molecular events such as changes in the degree of hydrophobic hydration of residues or the number of soft vibrational and rotational modes. However, before ΔC_p values are interpreted in molecular terms, particularly in terms of structural changes of the macromolecule, care must be taken to exclude other processes, e.g., protonation equilibria, as the source of an apparent ΔC_p. Our data permit us to make an estimate of the possible contribution of temperature-dependent protonation equilibria to ΔC_p. According to the equation (Alberty, 1969)

$$\left(\frac{\partial \Delta H}{\partial \mathrm{pH}}\right)_T = 2.3RT^2\left(\frac{\partial n}{\partial T}\right)_{\mathrm{pH}} \quad (6)$$

the isothermal pH dependence of the reaction enthalpy is proportional to the number of protons, n, produced per degree at constant pH. R is the gas constant and T the temperature in kelvin. By use of the values of Tables I and II $(\partial \Delta H/\partial \mathrm{pH})_T$ can be determined for 15, 25, and 35 °C. The results are −68 100, −48 200, and −56 700 J/mol of β_2, respectively. When these numbers are employed in eq 6, the resulting values for $(\partial n/\partial T)_{\mathrm{pH}}$ are −0.038, −0.028, and −0.031. Correspondingly, within the temperature range of 20 deg a maximum of approximately 0.76 proton is absorbed per β_2 subunit. Under the assumption of a large heat of ionization of 42 kJ/mol of

proton, the maximum contribution, which could stem from temperature-induced proton absorption equilibria, is −32 kJ/mol of β_2. This enthalpy corresponds to approximately 18% of the enthalpy change observed as a function of temperature at each of the three pH values. Therefore, it can be safely concluded that ionization equilibria are no major factor determining the magnitude of the ΔC_p observed for binding of PLP to β_2. The unusually large value of ΔC_p for complex formation of two PLP with β_2 is −9.1 kJ/(mol of β_2·K). In view of the insignificant role which proton equilibria play for the magnitude of ΔC_p, it is reasonable to interpret the large negative heat capacity change as reflecting major structural changes of β_2 as a result of binding of PLP. Although the molecular nature of the changes cannot uniquely be deduced from the thermodynamic parameter, the negative sign indicates that only a few processes such as tightening, a reduction in the degree of hydrophobic hydration, and an improvement in van der Waals interaction are consistent with the sign of ΔC_p (Sturtevant, 1977). Negative heat capacity changes of this magnitude usually have been obtained for association of proteins (Ross & Subramanian, 1981; Hinz, 1983), where the values range between 1 and 16 kJ/(mol·K), whereas for binding of small ligands to proteins values between 1 and 2 kJ/(mol·K) have been determined (Schmid et al., 1976; Niekamp et al., 1980). Only for the reaction of the substrate with the Leu-Ile-Val binding protein of *E. coli* a comparatively large ΔC_p of −9 kJ/(mol·K) at temperatures higher than 40 °C has been reported (Gaudin et al., 1980).

The unusually large ΔC_p value upon PLP binding to β_2 becomes even more striking when compared with the ΔC_p observed for binding of PLP to the native apoenzyme $\alpha_2\beta_2$. Formation of the $\alpha_2(\beta\text{-PLP})_2$ complex is associated with a heat capacity change of only −1.7 kJ/(mol of β_2·K). Such a difference in ΔC_p for the same reaction can be rationalized if the tightening and structuring influence of both the coenzyme and the α subunits on the conformation of the β subunits is taken into account. Actually, the different magnitude of ΔC_p could have been predicted on the basis of our previous experiments on the association of α and β_2 subunits in the absence and presence of the coenzyme PLP (Wiesinger et al., 1979). While the reaction of α subunits with β_2 subunits was found to be associated with a heat capacity change of −15 kJ/(mol of β_2·K), holoenzyme formation between α subunits and β_2 subunits presaturated with PLP, i.e., $(\beta\text{-PLP})_2$, involved the smaller ΔC_p of −9.6 kJ/(mol of β_2·K). Both these ΔC_p differences can be consistently interpreted, if one assumes that complex formation of β_2 with either two PLP or two α subunits induces conformational changes. Binding of the second ligand then occurs to a prestructured complex (β_2–PLP_2 or $\alpha_2\beta_2$) which consequently involves less structural rearrangement and therefore a smaller ΔC_p.

This interpretation is confirmed by measurements of the quenching of tryptophan fluorescence. The results of these experiments suggest that the α subunits stabilize a conformation of the β_2 subunit which is more similar to that of the $(\beta\text{-PLP})_2$ complex than to that of β_2 in the absence of PLP (Lane, 1983).

The tighter structure of the $(\beta\text{-PLP})_2$ complex, as compared to that of β_2, results also in an increased stability against thermal denaturation, as preliminary scanning calorimetry experiments show. Although we cannot distinguish between the two independently folding domains F_1 and F_2 that Högberg-Raibaud & Goldberg (1977a,b) have found by proteolytic cleavage of the β protomer, it is probable that PLP exerts its stabilizing influence primarily on the coenzyme binding domain F_1, as was suggested by Zetina & Goldberg (1980, 1982). However, since one molecule of PLP bound to the β_2 dimer is sufficient to protect both polypeptide chains against thermal inactivation (Zetina & Goldberg, 1980), strong interdomain interactions must be assumed to explain the protection of the unliganded domain. Similar results have been obtained with aspartate aminotransferase, where binding of PLP was found to increase the thermal stabilization of the enzyme by about 113 kJ/mol (Relimpio et al., 1981).

Besides the magnitude, two other aspects of the ΔC_p for PLP binding to both the β_2 subunit and the $\alpha_2\beta_2$ complex are noteworthy: ΔC_p is pH independent (Figure 4), and it is associated with the fast part of the overall reaction. These findings can be used to establish a sequence of identifiable subreactions for complex formation of PLP and β_2. The primary noncovalent attachment of the coenzyme to the protein via the charged phosphate group and the covalent formation of the imine linkage are fast (on a calorimetrically observable scale) as compared to the overall binding process. One of the two steps induces major structural rearrangements in the β_2 protein. The very slow phase in the time range of several minutes, seen in the calorimetric trace, must be ascribed to minor conformational changes, probably in the active center. This interpretation is supported by studies of Balk et al. (1981), which show that slow processes are responsible for the gain of enzymatic activity. Although these slow conformational rearrangements have no significant influence on the gross structure of the subunits and, therefore, do not show up in the ΔC_p, they contribute considerably to the reaction enthalpy, ΔH. These "localized" processes are responsible for the favorable energetic interactions between the coenzyme and the binding domain of β which, at 25 °C and higher pH values, render the overall ΔH a negative quantity by overcompensating the unfavorable intrinsic enthalpies of phosphate binding and Schiff base formation.

In summary, one can state that, due to the occurrence of slow sequential reaction steps involving enthalpic processes of opposite sign, it has been possible to determine thermodynamic parameters and to deduce a reaction mechanism for the binding of PLP to tryptophan synthase on the basis of calorimetric measurements alone. Four subreactions can be clearly inferred, although not all can be separated on the calorimetric time scale: (1) electrostatic interaction via the phosphate group; (2) Schiff base formation; (3) gross conformational changes [could also occur after (1)]: (4) slow localized conformational rearrangements in the time range of minutes. This reaction mechanism is in excellent agreement with a detailed kinetic analysis based on UV and fluorescence changes concomitant with PLP binding to β_2 (Bartholmes et al., 1980). In addition to the kinetic study, the calorimetric results provide clear evidence that major conformational changes are completed before attainment of full enzymatic activity.

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Registry No. PLP, 54-47-7; PAL, 66-72-8; PNP, 447-05-2; POL, 65-23-6; ε-aminocaproic acid, 60-32-2; tryptophan synthase, 9014-52-2.

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Energetic Adaptation of Ligand Binding to Subunit Structure of Tryptophan Synthase from *Escherichia coli*†

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ABSTRACT: The binding of indole and L-serine to the isolated α and β_2 subunits and the native $\alpha_2\beta_2$ complex of tryptophan synthase from *Escherichia coli* was investigated by direct microcalorimetry to reveal the energetic adaptation of ligand binding to the subunit structure of a multienzyme complex. In contrast to the general finding that negative heat capacity changes are associated with ligand binding to proteins, complex formation of indole and the α subunit involves a small positive change in heat capacity. This unusual result was considered as being indicative of a loosening of the protein structure. Such an interpretation is in good agreement with results of chemical accessibility studies (Freedberg & Hardman, 1971). Whereas the thermodynamic parameters of indole binding are not influenced by the subunit interaction, the large negative change in heat capacity of −6.5 kJ/(K·mol of β_2) measured for the binding of L-serine to the isolated β_2 subunit disappears completely when serine interacts with the tetrameric complex. These data demonstrate that the energy transduction pattern and therefore the functional roles of the substrates indole and L-serine vary strongly with the subunit structure of tryptophan synthase.

Specificity of coenzyme and substrate binding and catalytic efficiency of enzymes are highly correlated properties. Energy transduction upon complex formation with the specific ligands induces the energetic and structural changes necessary for the enzyme to reach the activated complex at physiological temperatures. Due to the linkage of all binding equilibria interactions between subunits of multimeric enzymes can be expected to influence the energetic pattern of substrate binding considerably (Pettigrew et al., 1982; Wiesinger et al., 1979).

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